Role of Ubiquitin Conjugating Enzyme E2K (UbE2K) in Regulating Adipogenesis

A Thesis
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Problem

Our laboratory focuses on the well-known tumor suppressor gene Phosphatase and tensin homolog (PTEN), lost in many cancers due to genetic or epigenetic changes. Importantly, reduced PTEN protein level, but not mRNA level correlates with poor outcome in some cancers, such as breast carcinoma. This suggests that post-translational mechanisms regulating PTEN protein stability could play a key role in disease progression.[1] The signaling pathways, components, and physiological conditions that regulate PTEN protein stability are not well studied and remain a major challenge in the field. Our laboratory undertook a global shRNA screen to identify factors that negatively regulate PTEN protein stability to understand the basic mechanism that regulates PTEN protein stability. One such factor that was identified in the shRNA screen is Ubiquitin Conjugating Enzyme E2K (UbE2K). As PTEN plays a critical role in regulating cellular homeostasis, it will be important to elucidate how PTEN protein turnover is regulated, and if *Ube2k* deletion will lead to restoration of PTEN level *in vivo*.

Background

PTEN

PTEN is a well-known tumor suppressor gene that possesses dual lipid and protein phosphatase activity. It is known that lipid phosphatase activity is directly associated with the role PTEN has as a tumor suppressor, while the importance of the protein phosphatase activities are gradually unraveling. PTEN regulates multiple cellular processes such as cell polarity and migration, growth and metabolism, cell-cycle progression.[2] PTEN dephosphorylates phosphatidylinositol (3,4,5)-triphosphate (PIP3) to PIP2, thus antagonizing the AKT pathway, as PIP3 normally activates AKT and its downstream signaling pathway. AKT activation promotes

cell survival, proliferation, growth, and glycogen metabolism (Fig. 1). PTEN is highly mutated in many cancers, and it has been suggested that genomic and non-genomic events lead to PTEN loss in cancer, which leads to uninhibited cell division through AKT signaling.[3]

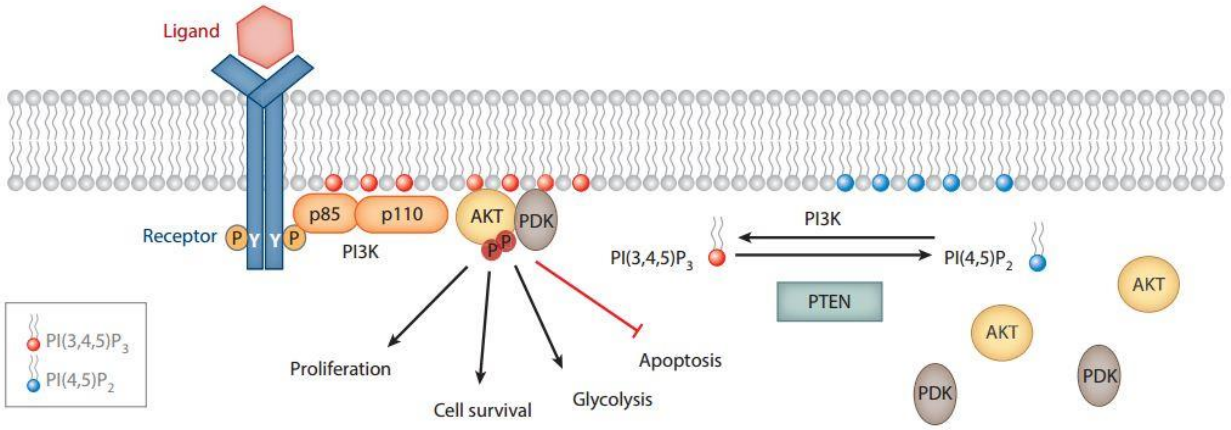

Figure 1 PTEN opposes the PI3K /AKT pathway. Worby et al., *Annu. Review Biochem.*, 2014

PTEN activity is regulated by many different mechanisms such as posttranslational modifications (including phosphorylation and ubiquitination), protein-protein interactions, transcriptional regulation, microRNAs, long noncoding RNAs, and antisense RNA.[2]

PTEN Phosphorylation:

PTEN (Fig. 2) is phosphorylated at its C2 domain, which upregulates PTEN activity and protein stability through increased membrane targeting and reduced polyubiquitination, respectively. PTEN is also phosphorylated at the C-tail domain, which inhibits the catalytic activity of PTEN and stabilizes the protein due to conformational changes.[2] It has also been noted that leptin signaling causes phosphorylation of PTEN, thus inactivating the phosphatase activity, and allowing the downstream signaling through the PI3K pathway.[2]

Though many studies focus on the mutations of *PTEN* in cancer, it is important to note that decreased PTEN expression, activity and cellular localization are just as important in promoting disease.[3]

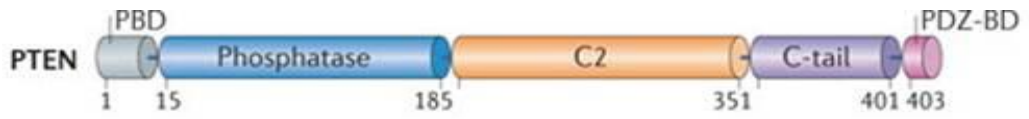

Figure 2 PTEN is comprised of 5 functional domains. Song M, et al., *Nature Reviews Molecular Cell Biology*, 2012

UbE2K

Our attempt to identify factors that negatively regulate PTEN protein stability unraveled UbE2K as a potential regulator of PTEN protein. UbE2K is a component of the ubiquitin-proteasome pathway and is specifically involved in polyubiquitination of target proteins, which promotes its degradation through the ubiquitin-proteasome pathway.[4] The ubiquitin-proteasome pathway consists of the ubiquitin activating enzyme, E1, the ubiquitin conjugating enzyme, E2, and the ubiquitin ligase, E3. The first step of the ubiquitin-proteasome pathway is the activation of the carboxyl group of ubiquitin by E1, which involves hydrolysis of one ATP molecule. Consequently, a thio-ester bond is formed between a ubiquitin molecule and E1. Following this, the activated ubiquitin molecule is transferred to the active site cysteine residue of an E2 enzyme. E2 then transfers the ubiquitin molecule to an E3 ligase through a thiol linkage; this E3 ligase in turn transfers the ubiquitin to a substrate protein that is bound to E3 ligases. Polyubiquitination of the substrate protein leads to its degradation through the proteasome pathway (Fig. 3).[5] E2 enzymes have the unique property of selecting the lysine (Lys) residue through which ubiquitin chains will be formed, a determinant of the cellular fate of the substrate. [6] Of the seven Lys residues in ubiquitin, polyubiquitination involving Lys48 leads to protein degradation, and

polyubiquitination through Lys63 regulates subcellular localization and activates signaling pathways.[7]

Previous studies have shown that E2 conjugating enzymes, like UbE2K, can transfer ubiquitin directly to target molecules if it is catalyzed by the RING-domain of E3 ligases.[8] UbE2K is specifically involved in polyubiquitination of target proteins, which promotes its degradation through the ubiquitin-proteasome pathway (Fig. 3).[9] The family of E2 enzymes contains a highly conserved ubiquitin-conjugating domain, which accommodate the ATP-activated ubiquitin or ubiquitin-like proteins through a covalently bonded thioester connected to its active-site cysteine residue. It is important to note that E2 enzymes partner with specific E3 enzymes, which determines the specificity of the downstream effector pathway on the substrate.[6]

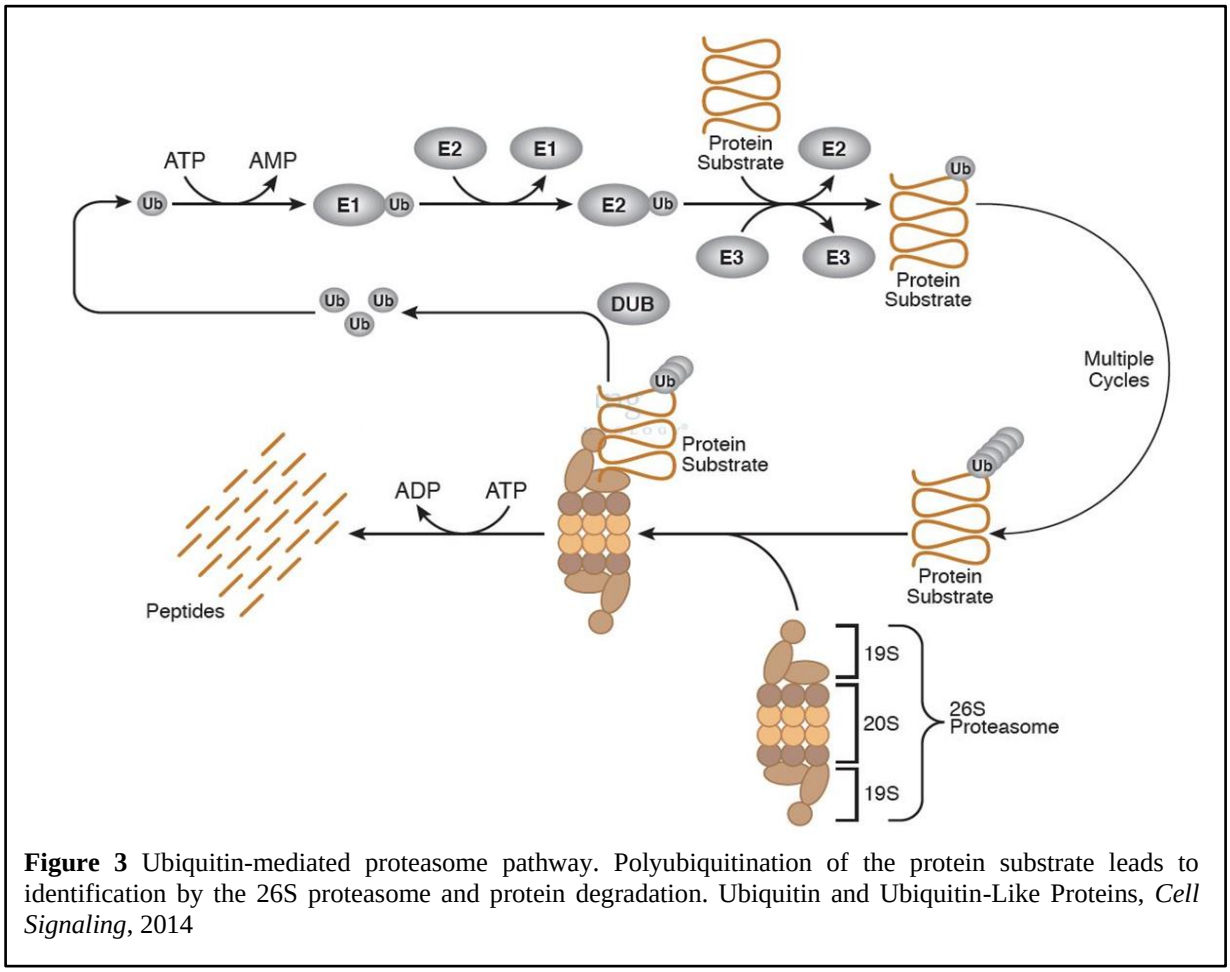

Figure 3 Ubiquitin-mediated proteasome pathway. Polyubiquitination of the protein substrate leads to identification by the 26S proteasome and protein degradation. Ubiquitin and Ubiquitin-Like Proteins, *Cell Signaling*, 2014

PTEN Ubiquitination:

Two lysine residues, Lys289 and Lys13, have been identified on PTEN that are ubiquinated and regulate the nuclear localization of PTEN. Together, these two residues regulate PTEN import.[10] Additionally, E3 ligases such as WWP2[11], NEDD4[12], MKRN1[12] have been identified to promote PTEN degradation. Recently a deubiquitinase USP13, has been shown to protect PTEN degradation by deubiquitinating PTEN. All of these studies imply that PTEN protein stability is regulated by the ubiquitin-proteasome pathway.[13]

Adipogenesis

There are two different types of adipose tissue in mammals: white adipose tissue (WAT) and brown adipose tissue (BAT). While WAT is primarily dedicated to energy storage, BAT plays a critical role in energy expenditure and heat generation. This energy expenditure is mediated primarily through mitochondrial activity.[14] Fatty acid oxidation occurs inside the mitochondria, and is the process by which fatty acids are broken down to yield acetyl-CoA, which then enters the citric acid cycle. Other important components of fatty acid metabolism are specialized proteins, such as uncoupling protein 1 (UCP1). UCP1 uncouples oxidative phosphorylation and ATP generation by collapsing the electron gradient to generate heat instead.[13] Conversely, amount of WAT regulates systemic metabolism, which has been implicated in various metabolic dysfunctions such as hyperglycemia, hyperlipidemia, diabetes, liver disease, etc. BAT is of key importance during the neonatal period for temperature control.[15]

An important mediator of adipogenesis is peroxisome proliferator-activated receptor gamma (PPARγ) – a nuclear hormone receptor that is necessary for adipogenesis by regulating lipid storage, lipid synthesis, and glucose sensing. It has been demonstrated that PPARγ is

expressed even before adipocyte tissue development begins; PPARγ is also expressed in adipose stem cells and plays a key role in stem cell proliferation and cell fate. These findings lead one to believe that PPARγ is also involved in early adipose lineage development.[14]

PTEN and Adipogenesis

As stated previously, the major role of PTEN is to inhibit the PI3K pathway. Several growth factors, such as insulin-like growth factors and insulin, activate the PI3K pathway and its downstream signaling events, including activation of Akt . This activation of Akt induces a complex cascade of reactions which leads to FOXO1 phosphorylation and its retention in the cytoplasm, thereby inhibiting its activity. It is important to note that the Foxo1 transcription factor along with the transcriptional co-activator, PGC1α, regulate the transcription of UCP1 protein. Increase in PTEN level will lead to inhibition of Akt, which in turn will activate FOXO1. PGC1α is expected to activate UCP1 transcription, thereby uncoupling oxidative phosphorylation and ATP generation in the mitochondria (Fig. 4).

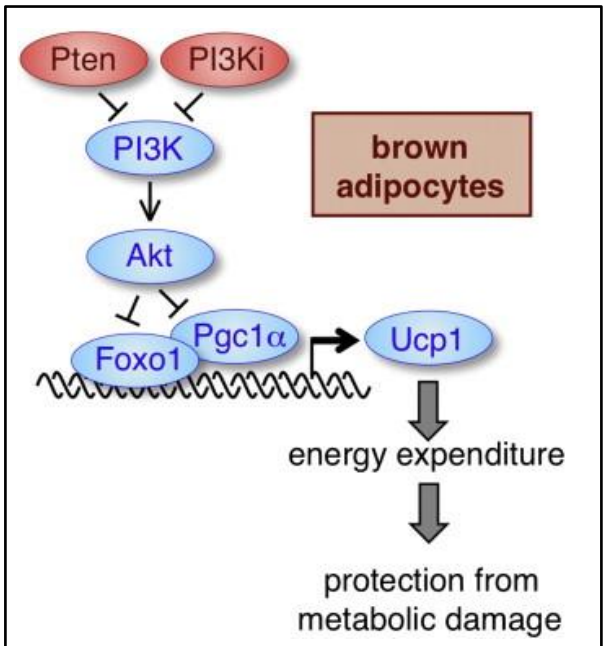

Figure 4 Role of PTEN in the metabolic pathway utilized in brown adipose tissue. Ortega-Molina et al. *Cell Metabolism*, 2012

In a recent study, Ortega-Molina et al. (2012) generated *Pten* transgenic (*Pten*^{tg}) mice by inserting Bacterial Artificial Chromosome clone (127kb) containing complete murine PTEN gene. The transgenic mice showed a 2-fold increase in PTEN level. The *Pten*^{tg} mice have low body fat, increased energy expenditure, lower serum leptin and cholesterol levels, smaller

adipocytes, dense and intensely colored BAT, and high uncoupling protein 1 (UCP-1) in BAT.

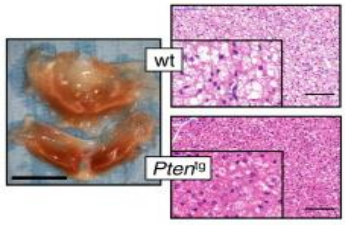

Figure 5 BAT phenotype in WT and PTEN^{tg} mice. Ortega-Molina et al., *Cell Metabolism*, 2012

UCP-1 uncouples mitochondrial oxidation and ATP production, thus mediating energy dissipation in BAT. The high amounts of UCP-1 in BAT parallels the dense phenotype observed in the BAT, indicating high BAT activity (Fig. 5).[16]

Significance

Not much is known about the factors that regulate PTEN protein stability; however, it has been shown that PTEN protein plays a critical role in regulating cellular homeostasis. Our study has identified several negative regulators of PTEN. One such regulator is UbE2K, which could be one of the key factors regulating its degradation. PTEN is highly mutated in many cancers, but there is still much to learn about how PTEN is regulated. This study aims to further investigate the mechanism mediating PTEN protein stability by studying the relationship between UbE2K and PTEN.

Hypothesis

We hypothesize that depletion of UbE2K will increase PTEN protein expression, both *in vitro* and *in vivo*. Based on the previously published studies[13], we hypothesize that adipocyte specific *Ube2k* deletion will lead to an increase in PTEN level, thereby causing high energy expenditure and a significant reduction in adipose tissue. Alteration in the mammary fat pad along with visceral fat depot could affect mammary gland development. In the future, we plan to study the role of UbE2K and PTEN in cancer and obesity to potentially find if the reciprocal relationship between the two has any effect specifically on mammary tumorigenesis.

Specific Aims

Specific Aim 1: *Investigate if depletion of UbE2K causes increased PTEN protein levels*

UbE2K was depleted in MCF10A, normal mammary epithelial, cells using lentivirus coding for *UbE2K* targeted shRNAs. Additionally, mice with conditional knockout alleles for *Ube2k* (EUCOMM, using the Cre-Lox recombination system in the Leone Lab) were generated to further understand the role of this gene in a more complex organism.

Specific Aim 2: *Investigate the in vivo phenotypes associated with adipocyte-specific Ube2k deletion*

We observed a significant reduction of white adipose tissue (WAT) and brown adipose tissue (BAT) in the whole-body *Ube2k* knockout mice, and hypothesized that increased Pten levels in *Ube2k* KO mice affect the normal development of adipose tissue. Ube2k was knocked out in the adipocytes using an adipocyte-specific cre (Adiponectin-Cre; Adipoq-Cre). These mice were then compared to the whole-body knockout mice to study how Ube2k regulates adipogenesis and mammary land development in adult mice.

Study Design

Specific Aim 1A: *Investigate if depletion of UbE2K causes increased PTEN protein levels in vitro*

MCF10A, normal mammary epithelial cells, will be transduced with lentivirus coding for UbE2K targeted shRNAs to assess whether depletion of this putative PTEN regulatory gene affects PTEN *in vitro*. MCF10A cells transduced with scrambled shRNA will be used as negative control. PTEN and UbE2K mRNA levels in the UbE2K shRNA transduced MCF10A cells (MCF10A/shUbE2K) will be assessed by qRT-PCR, protein levels will be assessed by western blotting and compared with that in the negative control samples.

Specific Aim 1B: *Investigate if depletion of UbE2K causes increased PTEN protein levels in vivo*

Ube2k Neohypomorph (*Ube2k*$^{neo/neo}$) mice will be bred with *ACTFLPe* mice to delete the lacZ-*neo* artificial cassette and generate the *Ube2k* floxed mice (*Ube2k* $^{fl/fl}$*).* We will generate whole body knockout mice (KO) for *Ube2k* by breeding *Ube2k* $^{fl/fl}$ mice with Sox2-Cre mice to study the phenotype of the KO mice

Specific Aim 2A: *Determine if adipocyte-specific knockout of Ube2k mimicks the whole-body knockout phenotype*

Since there is a significant reduction of fat in the whole-body *Ube2k* KO mice, we were curious to see if UbE2K played an integral role in adipogenesis. *Adipoq-cre; Ube2k* $^{fl/fl}$ mice will be generated by breeding *Ube2k* $^{fl/fl}$ mice with Adipoq-cre mice to study their life span, body weight, and histological nature of the fat pads will be compared to those of the whole-body *Ube2k* KO mice (*Ube2k*$^{-/-}$).

Specific Aim 2B: *Study how mammary gland development is affected in Adipoq-Cre; Ube2k*$^{fl/fl}$ *mice*

Mammary glands of *Adipoq-cre; Ube2k* $^{fl/fl}$ and *Adipoq-cre; Ube2k* $^{fl/fl}$ mice will be harvested at one and three months of age to assess any histological changes in mammary gland development.

Materials and Methods

Preadipocytes:

Isolation: The intrascapular (ISCW) and inguinal (IGW) fat pads will be minced in sterile phosphate buffered saline (PBS) buffer, and digested using collagenase at 37^{o}C while being vortexed for 100 seconds every 5 minutes for a total of 40 minutes. The digested tissue will then be filtered through 100um filters into Eppendorf vials and centrifuged the vials at room temperature at 1500rpm for 5 minutes. Upon removing the supernatant, the pellet will be resuspended in 1mL Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum, 20mM HEPES buffer solution, and 1X penicillin/streptomycin following standard protocol.[17]

Immortalization: The preadipocytes will be immortalized by infecting the cells with the retroviral vector pMSe-SV40T that encodes for the SV40T antigen and selecting them with G418.

Differentiation: Adipocyte differentiation is a 10-day time course. On day 1, the preadipocytes will be seeded into 6-well plates in 4mL differentiation media (DMEM, 10% FBS, 2nM insulin, 1uM T3 filter sterilized) at a density to be confluent in four days. On day 4, cells are induced in 4mL induction media (Differentiation media, 0.125M indomethacin, 2mg/mL dexamethasone, 0.5M 3-isobutyl-1-methylxanthine (IBMX)). On day 6 and 8, the cells are put back into 4mL of fresh differentiation media. By day 10, the cells should be fully differentiated.

Protein: The cells will be lysed using RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP-40, 0.05% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate). These protein extracts will then be separated by a 10% reduced SDS-PAGE gel, and the membrane will be probed with PTEN, UbE2K, and GAPDH specific antibodies and analyzed using *Li-COR* imaging.

RNA: RNA will be isolated using Trizol (Life Technologies). cDNA will be made using High Capacity cDNA kit (Applied Biosystems). Real-Time PCR will be performed using SYPR Green PCR Master Mix (Applied Biosystems).

Immunohistochemistry: Tissues will be fixed in 4% paraformaldehyde for 48 hours and sectioned into 4 μm sections. Slides will then be stained with, Haemotoxylin and Eosin, HIP2 (#8226S), PTEN (#9559L), and Ki-67 (#ab16667) antibodies.

Oil Red O:

Tissue: P2 pups will be sacrificed and fixed in formalin for 48 hours. Tissue will be embedded in OCT and sent for sectioning into 10 um frozen sections. The frozen sections will first be washed in tap water for three minutes. They will then be stained with filtered working Oil Red O (Stock: 0.5% Oil Red O in 500 mL isopropanol. Working solution: 60% Oil Red O stock, 40% distilled water) solution. Following this first stain, the sections will be washed again in tap water for three minutes. The sections will then be stained with Harris hematoxylin stain for three minutes and then washed in tap water for 3 minutes. Finally, the sections will be placed in ammonia water for 45 seconds, washed in tap water for 3 minutes, and mounted with aqueous mounting media.

Cells: Once cells are differentiated, plates will be rinsed with PBS. Cells will be fixed by covering with PBS and formalin for 15 minutes at room temperature. Once cells are fixed, they will be stained with Oil Red O working solution (Stock: 0.5% Oil Red O in 100mL isopropanol. Working solution: 60% Oil Red O stock, 40% distilled water) for one hour at room temperature. Following this, the cells will be rinsed several times with distilled water to remove excess stain and precipitate. Finally, the dishes will be allowed to air dry.

Results

Knockout of UbE2K in mammalian cell lines causes increased PTEN protein, not mRNA, levels

We observed a marked reduction in UbeE2K mRNA and protein level in the MCF10A/shUbE2K cells, demonstrating efficient knockdown of UbE2K in these cells. We also observed a significant increase in PTEN protein levels in the UbE2K depleted cells compared to the control cells. There was no significant change in PTEN mRNA levels. Similar stabilization and increased half-life of PTEN protein was observed in human mammary fibroblast depleted of UbE2K when treated with cycloheximide, an inhibitor of protein synthesis. These data suggest that knockdown of UbE2K increases the stability of PTEN protein, and not PTEN mRNA levels (Fig. 6).

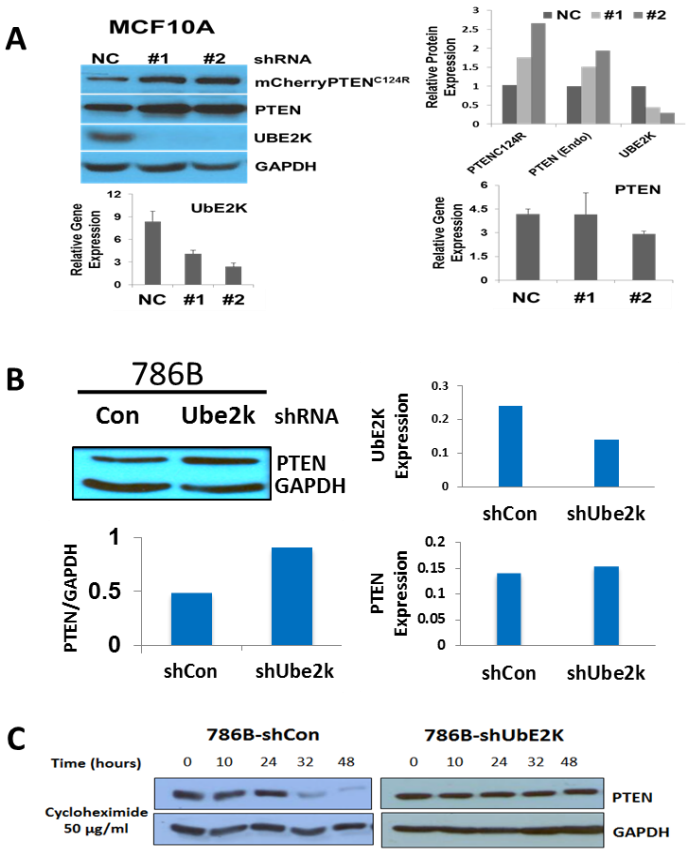

Figure 6 A) Knockdown of MCF10A cells using two non-overlapping shRNAs targeted towards UbE2K mRNA resulted in increased both PTEN^{C124R} and endogenous PTEN protein levels compared to a scrambled control. There were no significant changes in PTEN mRNA levels. B) Knockdown of UbE2K in patient-derived mammary fibroblasts recapitulated these results. C) Treatment of these fibroblasts with cycloheximide revealed an increase in PTEN stability upon depletion of UbE2K.

Whole-body knockout of Ube2k in vivo causes significant developmental defects in mice

We have generated mice with conditional knockout alleles for *Ube2k* (EUCOMM, using the Cre-Lox recombination system in the Leone Lab, Fig. 7). We observed marked developmental

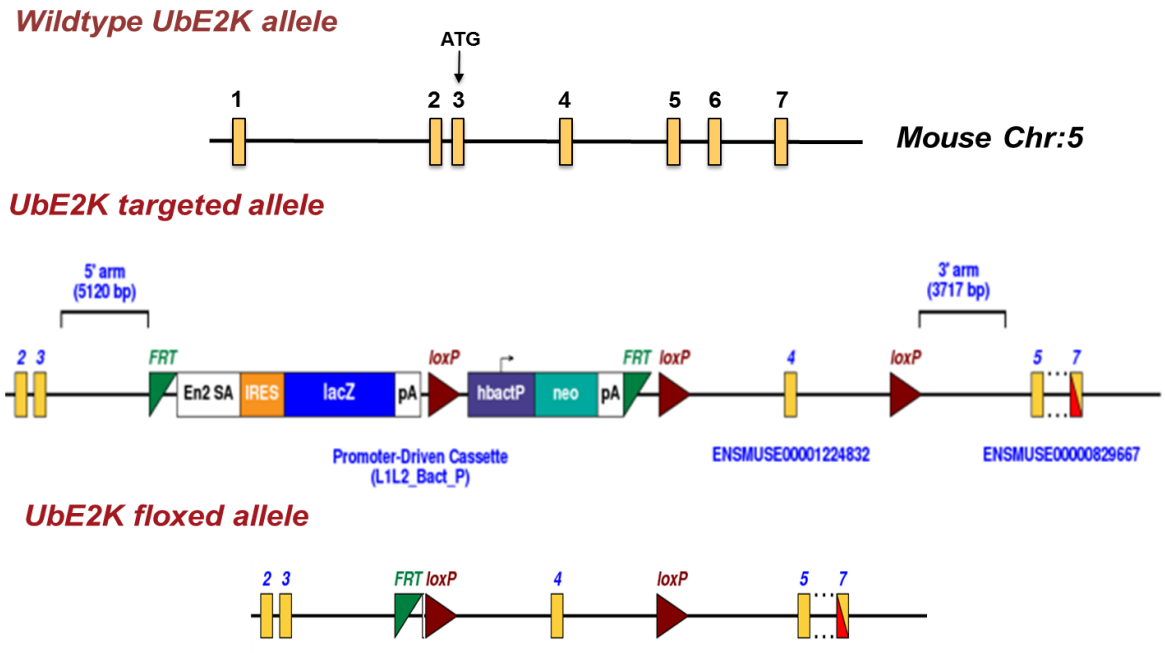

Figure 7 Targeted vector for UbE2K allele, LoxP sites were introduced flanking exon 4 of the UbE2K gene and a LacZ-Neo Cassette was introduced between exons 3 and 4 flanked by FRT sites.

defects in *Ube2k* KO mice. The KO mice are smaller at birth (Fig. 8, 9A, B) and die within postnatal day 2 (P2). According to Chi-square statistical analysis, these mice are not born in a Mendelian ratio. We harvested pups between postnatal day 1 and 2 and tissue sections were analyzed for gross histological changes after staining with Haematoxylin and Eosin (H&E). To confirm the efficiency of Sox2-Cre and the effect of UbE2K downregulation *in vivo*, using immunohistochemistry, we stained tissue sections of P2 mice with UbE2K and PTEN specific antibodies (Fig. 9C). Immunohistochemical analysis showed complete deletion of *Ube2k* in the KO mice. Analysis of the P2 pups showed an underdevelopment of the WAT and condensed BAT in the *Ube2k* KO mice compared to their WT littermates (Fig. 10). To investigate the extent to which the WAT is underdeveloped, we stained frozen tissue sections with Oil Red O stain to

detect fat globules in the tissue. We observed a complete lack of fat globules in the *Ube2k* KO mice in contrast to its abundance in WT littermates (Fig. 11). These *Ube2k* KO mice die between postnatal day 1 and 3 (P1 and P3).

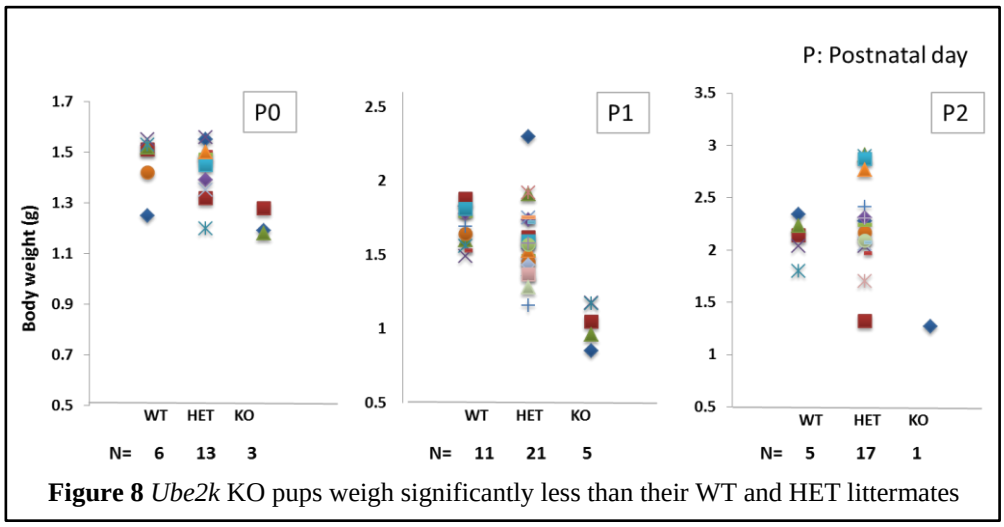

Figure 8 *Ube2k* KO pups weigh significantly less than their WT and HET littermates

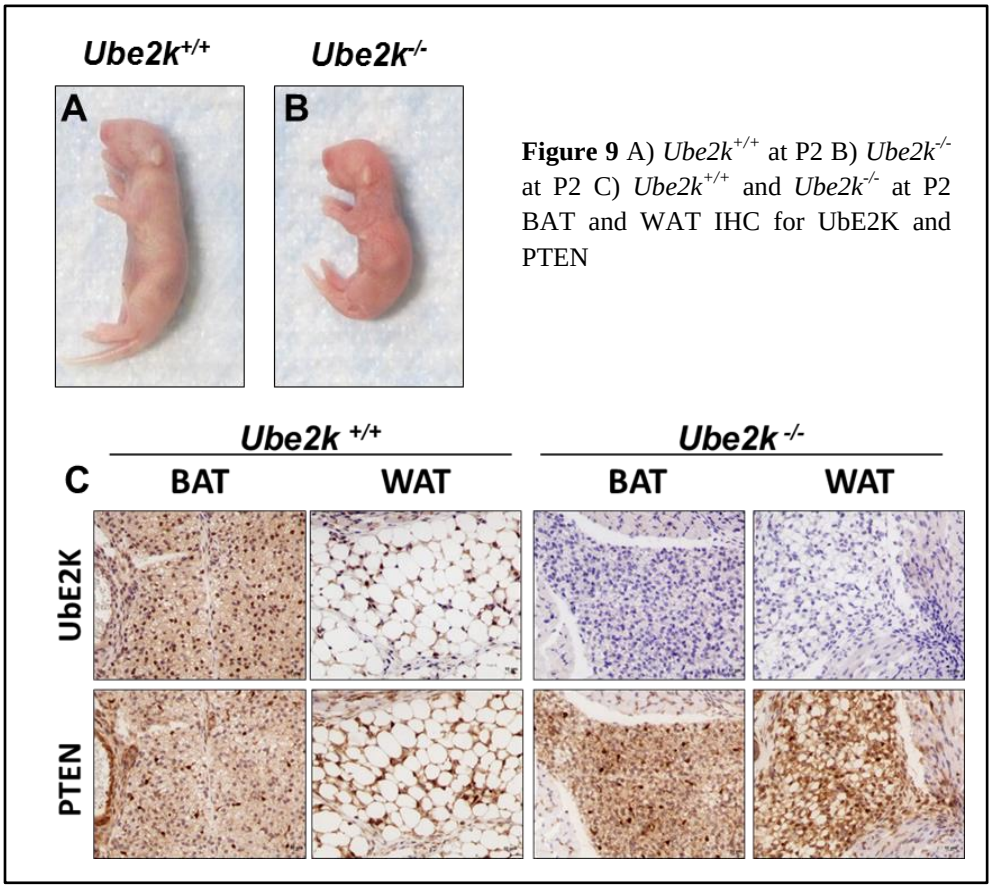

Figure 9 A) *Ube2k*$^{+/+}$ at P2 B) *Ube2k*$^{-/-}$ at P2 C) *Ube2k*$^{+/+}$ and *Ube2k*$^{-/-}$ at P2 BAT and WAT IHC for UbE2K and PTEN

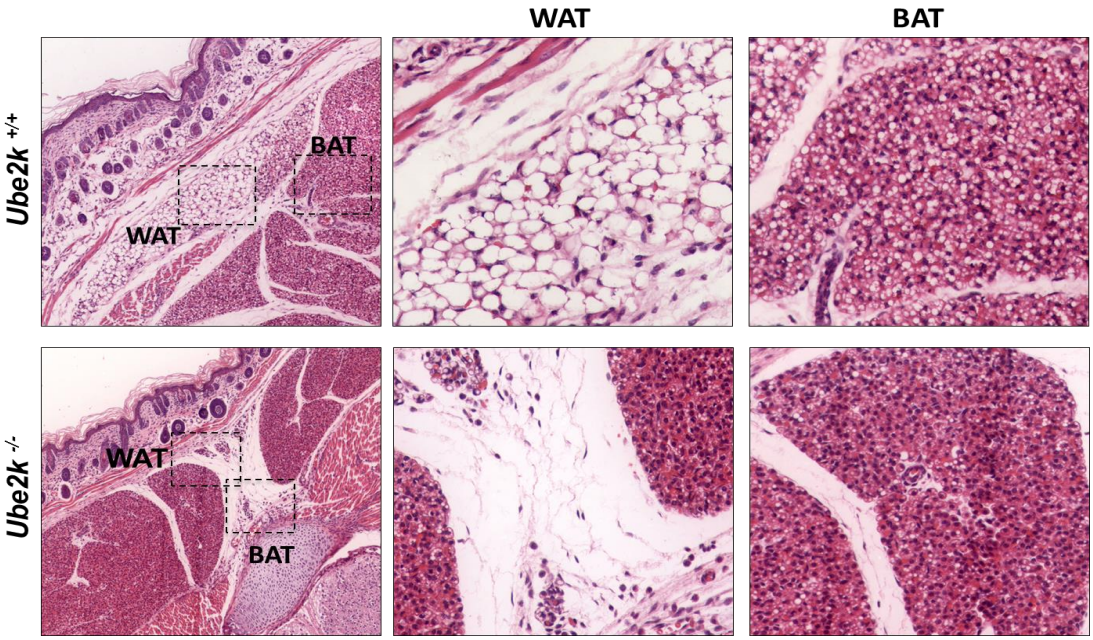

Figure 10 WAT and BAT of *Ube2k*$^{+/+}$ and *Ube2k*$^{-/-}$ at P2 stained with H&E

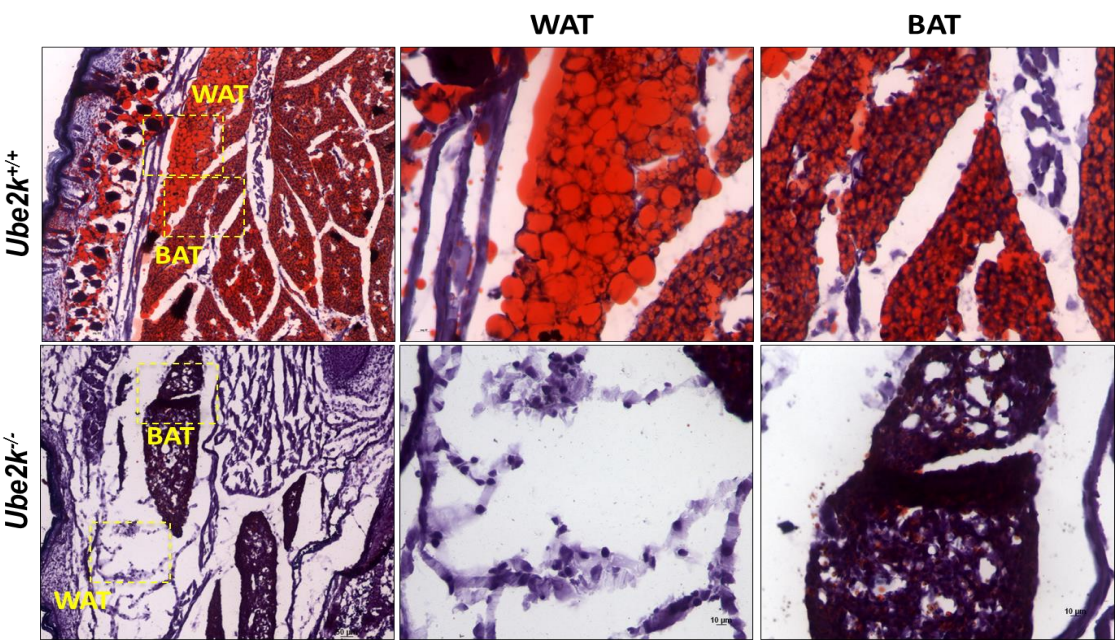

Figure 11 WAT and BAT of *Ube2k*$^{+/+}$ and *Ube2k*$^{-/-}$ at P2 stained with Oil Red O detected neutral fat globules in the WT, but not KO mice

Ube2k is completely knocked down in the adipose tissues and preadipocytes isolated from the KO mice

WAT/ BAT: ISCW and IGW fat pads, comprised of preadipocytes that will differentiate into BAT and WAT, respectively, (Fig. 12A) were harvested from P2 *Ube2k* WT and KO mice, and RNA was isolated from these tissues. PTEN and PPARγ mRNA levels were assessed by qRT-PCR. According to these results, both PTEN and PPARγ mRNA levels increase in the KO mice compared to their WT littermates (Fig. 12B, C).

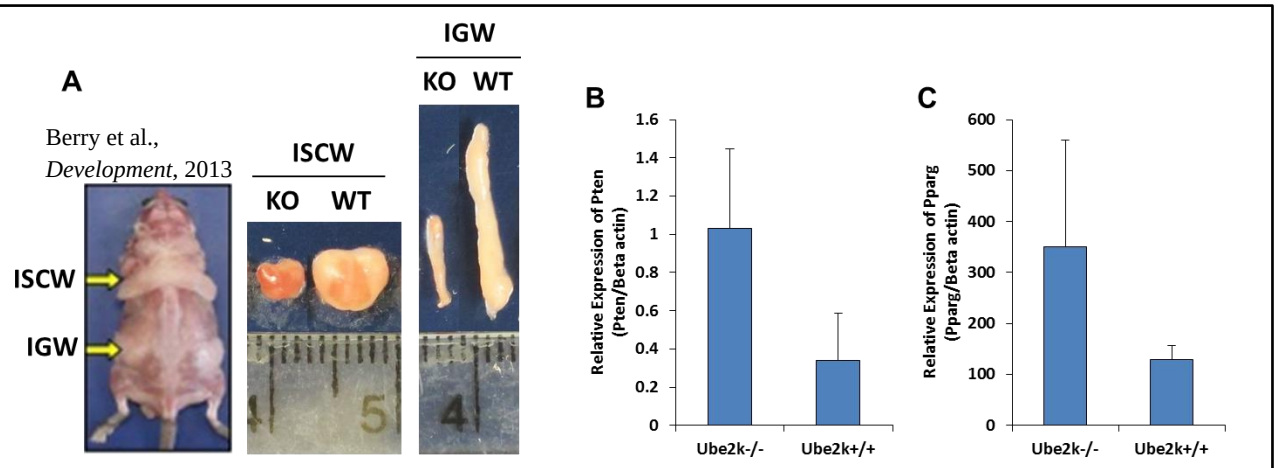

Figure 12 A) There is a significant decrease in ISCW and IGW fat pad volumes in *Ube2k*$^{-/-}$ compared to *Ube2k*$^{+/+}$ mice at P2. B) *Ube2k* deletion resulted in an increase in PTEN mRNA. C) *Ube2k* deletion resulted in a decrease in PPARγ mRNA levels

Preadipocytes: ISCW and IGW fat pads were harvested from P2 *Ube2k* WT and KO mice and made into preadipocyte cell lines. Once the preadipocytes were immortalized, the cells were harvested for protein and RNA. Expression of Ube2k and PTEN protein and RNA levels in the preadipocytes were assessed by western blot and qRT-PCR, respectively. There is significant depletion of UbE2K protein however no significant difference in PTEN protein in *Ube2k* KO preadipocytes compared to that derived from the wild type littermates (Fig. 13A); PTEN mRNA levels increased in the *Ube2k* KO preadipocytes as compared to their wildtype littermates (Fig.13B).

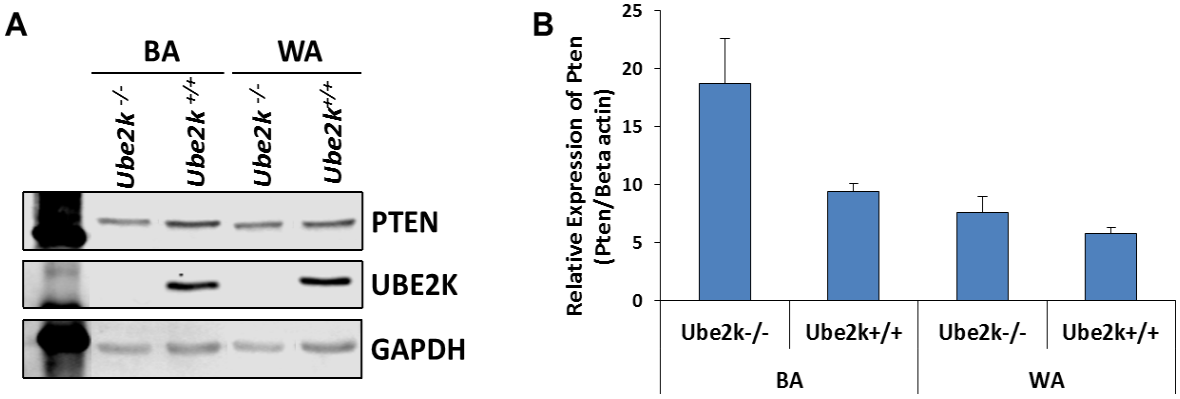

Figure 13 A) Western blot analysis shows complete knockdown of *UBE2K* protein and no change in *PTEN* protein. B) qPCR analysis increased *Pten* mRNA

Differentiation of the preadipocytes to adipocytes in vitro presented no difference in fat globule formation between Ube2k WT and KO adipocytes

To assess if adipocyte differentiation is regulated by UbE2K, we transduced *Ube2k*$^{fl/fl}$ preadipocytes into adipocytes with either p-Babe-Hygro (pBH) empty vector and pBH-Cre to delete *Ube2k*. We next differentiated both cell lines to adipocytes and stained the cells with Oil O Red. The preadipocytes differentiated into adipocytes in both the control and experimental cell lines; however, there was no difference in fat globule staining in either line. (Fig. 14).

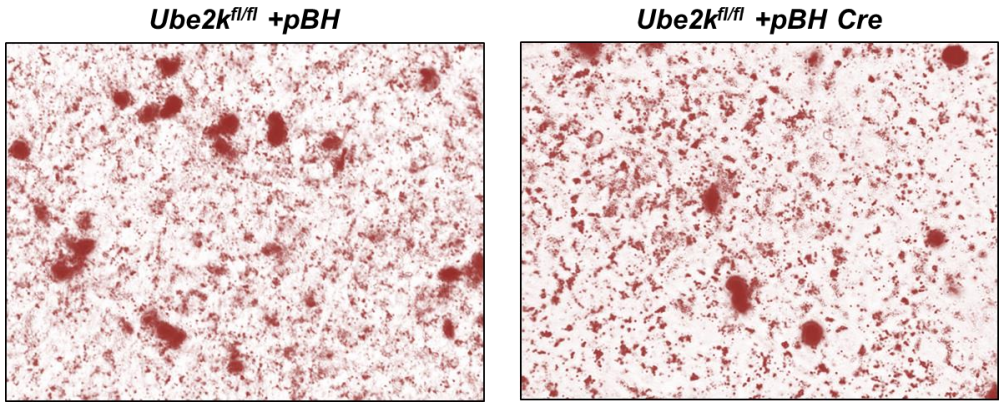

Figure 14 The Oil O Red staining displays neutral fat globules even in the cells lacking *Ube2k*

Adipocyte-specific knockout of Ube2k in vivo does not mimick the same phenotype as whole-body knockout mice

Hypothesizing that the whole-body *Ube2k* KO mice die at P2 was due to their lack of ability to properly regulate body heat as a result of significantly reduced fat pad volume, we were interested to see if knocking out *Ube2k* specifically in the adipocytes would induce the same phenotypes. The experimental *Adipoq-ce; Ube2k* $^{fl/fl}$ mice were born in a Mendelian ratio, there was no post-natal lethality, unlike the whole-body KO mice, and had no significant differences in body weight compared to control mice littermates (Fig.15A). To investigate if there was any

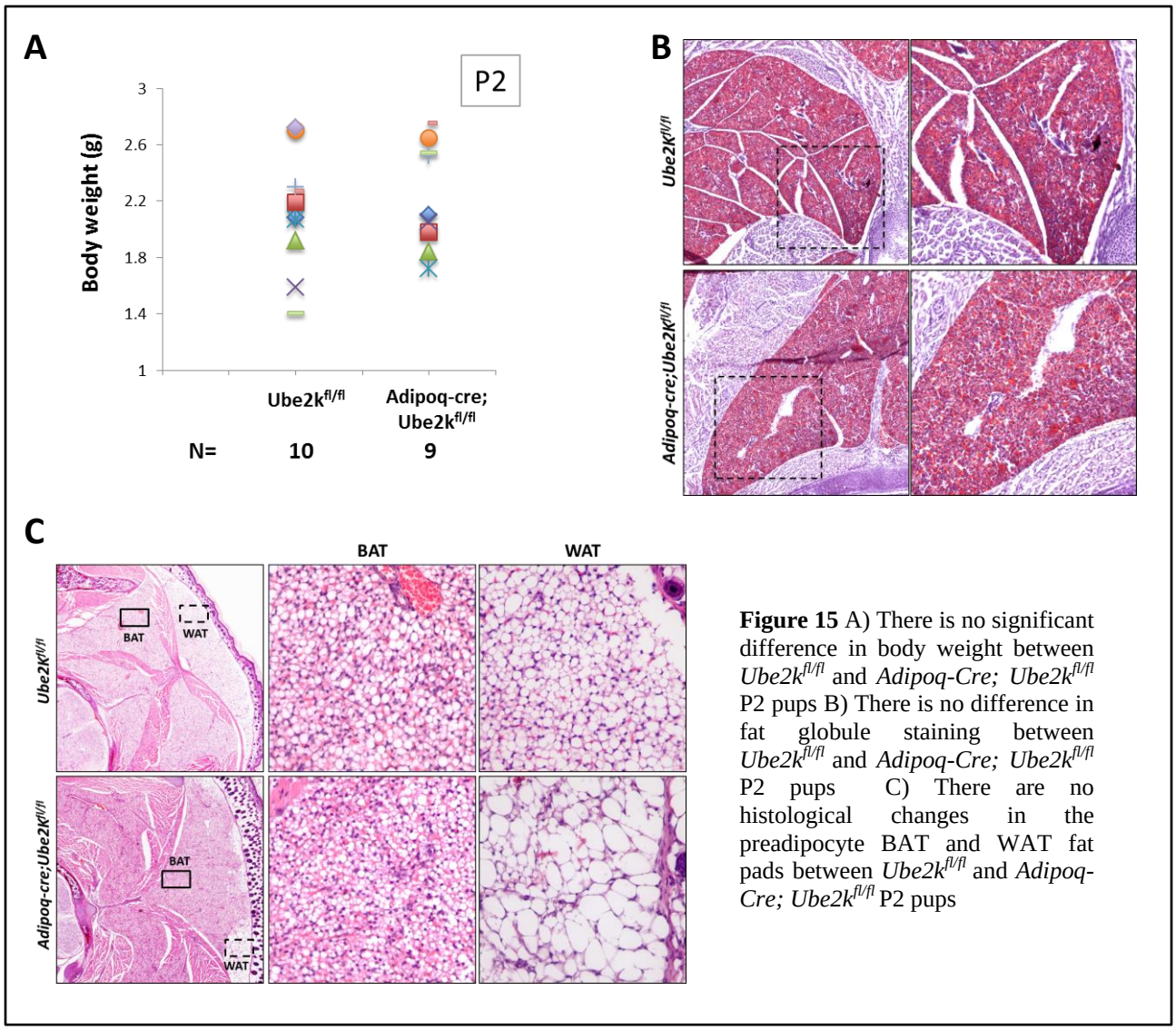

Figure 15 A) There is no significant difference in body weight between *Ube2k*$^{fl/fl}$ and *Adipoq-Cre; Ube2k*$^{fl/fl}$ P2 pups B) There is no difference in fat globule staining between *Ube2k*$^{fl/fl}$ and *Adipoq-Cre; Ube2k*$^{fl/fl}$ P2 pups C) There are no histological changes in the preadipocyte BAT and WAT fat pads between *Ube2k*$^{fl/fl}$ and *Adipoq-Cre; Ube2k*$^{fl/fl}$ P2 pups

underdevelopment of the fat pads, we stained frozen tissue sections with Oil Red O stain to detect fat globules in the tissue. We did not observe any difference in fat globule staining in *Adipoq-ce; Ube2k* $^{fl/fl}$ mice compared to *Ube2k* $^{fl/fl}$ mice. Additionally, there was no histological difference in the BAT between the experimental and control mice; though there appears to be histological changes in the WAT, we have not observed underdevelopment of the fat pads at P2 as seen in the whole-body *Ube2k* KO pups (Fig. 15B).

Adipocyte-specific knockout of Ube2k in vivo induces increased branching in the mammary gland

We observed an underdevelopment of mammary fat pads in P2 whole-body *Ube2k* KO mice. Because of this, we were interested to see if and how the development of the mammary gland would be altered in mice with *Ube2k* knocked out specifically in the adipocytes. In one-month old experimental mice, mammary gland branching extends beyond the lymph node, whereas in control mice littermates, the branching of the mammary gland does not progress as far (Fig. 16A, B). Additionally, in three-month old experimental mice, there is an increase in branching compared to the wild type littermate controls (Fig. 16C, D). The mammary glands were stained with Ki-67, a cellular proliferation marker, and there was no significant difference to be found in Ki-67 levels between experimental and control mice (Fig. 17).

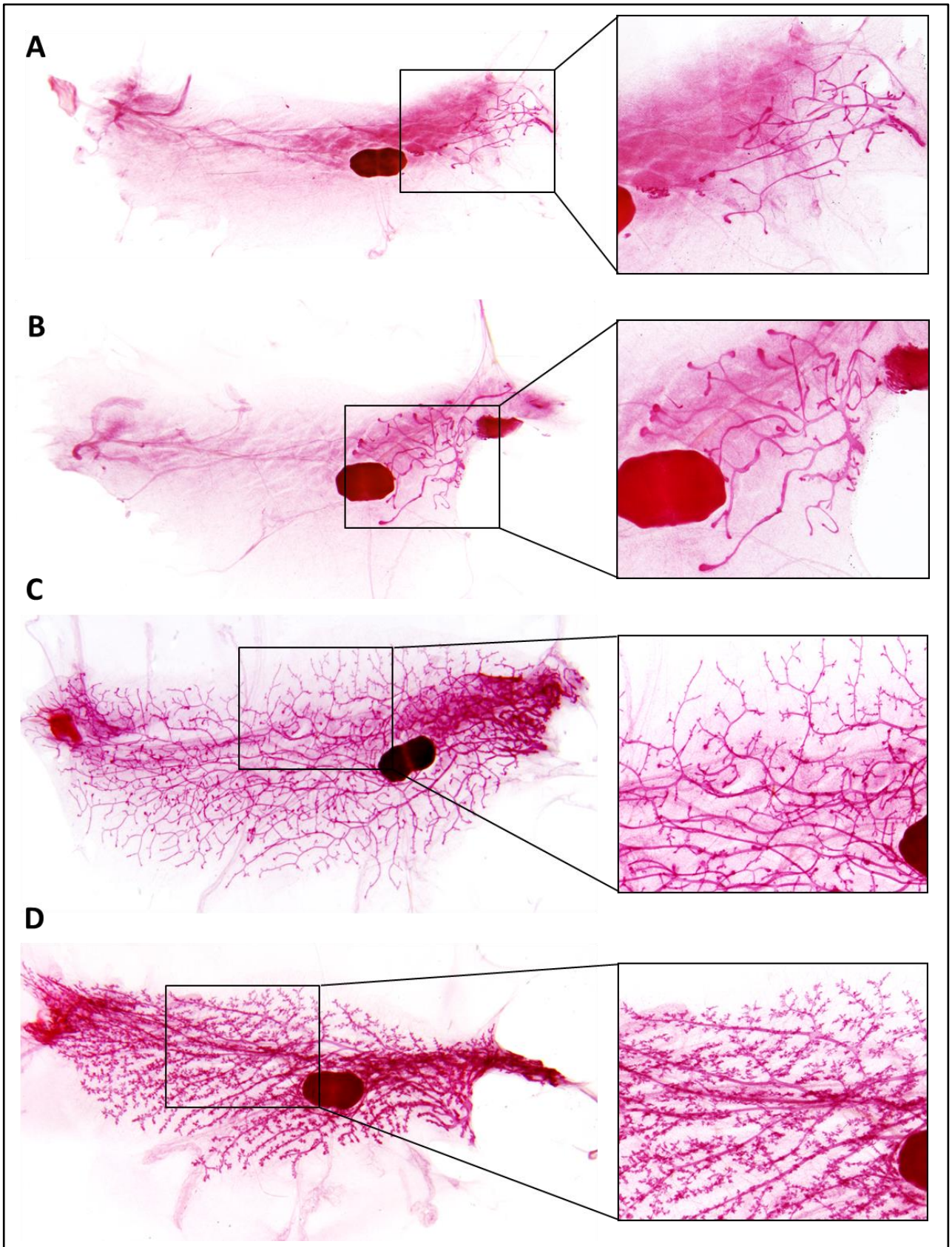

Figure 16 Whole mounts of mammary glands from A) One month old *Ube2k*$^{fl/fl}$ B) One month old *Adipoq-cre; Ube2k*$^{fl/fl}$ C) Three month old *Ube2k*$^{fl/fl}$ D) Three month old *Adipoq-cre; Ube2k*$^{fl/fl}$. Figures A-D stained with H&E to show histological nature of the mammary glands. *Adipoq-cre; Ube2k*$^{fl/fl}$ mice show further progression and increased branching in the mammary gland as compared to *Ube2k*$^{fl/fl}$ littermates.

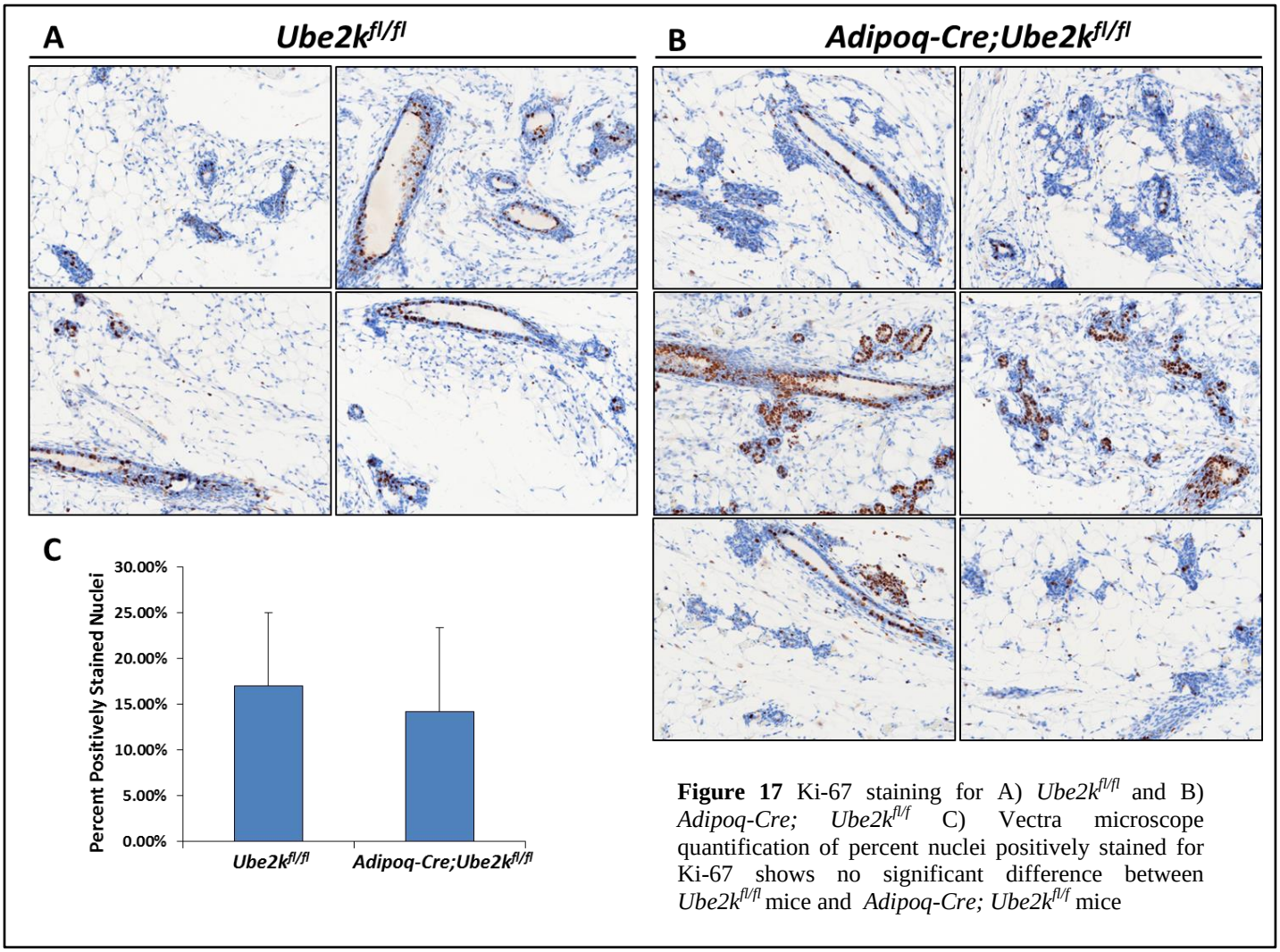

Figure 17 Ki-67 staining for A) *Ube2k*$^{fl/fl}$ and B) *Adipoq-Cre; Ube2k*$^{fl/f}$ C) Vectra microscope quantification of percent nuclei positively stained for Ki-67 shows no significant difference between *Ube2k*$^{fl/fl}$ mice and *Adipoq-Cre; Ube2k*$^{fl/f}$ mice

Discussion

UbE2K was identified through a global shRNA screen as a negative regulator of PTEN; we investigated the link between PTEN and UbE2K to understand the basic mechanism by which UbE2K influences PTEN protein stability.

It was seen in normal mammalian epithelial cells that knockdown of UbE2K *in vitro* causes an increase in PTEN protein levels, but no change in mRNA levels. When *Ube2k* was knocked out *in vivo*, the mice were not born in a Mendelian ratio, had a significant decrease in body weight, and did not live past P3. Much to our surprise, we noticed a phenotype in the preadipocyte fat pads where there was a significant decrease in fat pad volume in the *Ube2k* KO mice as compared to their WT littermates. Upon observing this phenotype, we hypothesized that UbE2K plays a role in adipogenesis. It is interesting to note that there is an increase in *Pparγ* mRNA levels in *Ube2k* KO BAT as compared to the WT littermates. Pparγ is thought to play an integral role in adipogenesis.[13] It has been seen that Pparγ increases adiponectin levels[18], and adiponectin is believed to enhance glucose sensitivity through fatty acid oxidation and inhibiting hepatic glucose production.[19] It is possible that increasing fatty acid oxidation enhances energy expenditure, thus depleting the fat storage. This information leads us to believe that UbE2K may also have a role in this relationship between PPARγ and adiponectin.

To further understand the function of UbE2K in adipogenesis, we generated preadipocyte cell lines from the ISCW and IGW preadipocyte fat pads in *Ube2k* KO and WT P2 pups. Upon differentiating the preadipocytes into adipocytes, we did not observe any difference in the differentiation between the KO and WT cells, nor did we notice any difference in fat globule staining once stained with Oil Red O. This data prompted us to believe that UbE2K may not directly be a critical regulator of adipogenesis, but rather it may a contributing factor to one of the various pathways that regulate adipogenesis.

Pursuing this idea, we knocked out *Ube2k* specifically in the adipocytes of mice using Adipoq-cre. These mice were born in Mendelian ratio, did not display differences in body weight, and did live beyond P3. Additionally, there were no histological changes in the preadipocyte ISCW and IGW fat pads as noted in the whole body KO mice. Since there was a significant underdevelopment of the mammary fat pads in the whole body KO mice, we studied the mammary glands of these Adipoq-cre mice. We observed branching that extended past the lymph node in the mammary glands of the one-month old experimental mice but not the control mice. There was also increased branching in the mammary glands of the three-month old experimental mice and not in the control littermates. This phenotype has huge implications in the scope of breast cancer, as invasion is a major element in tumorigenesis.

Studies have shown that one of the roles of the fat pad in the mammary gland is to inhibit glandular branching.[20] It has also been noted that unrestricted branching can eventually lead to tumorigenesis. The very nature of mammary gland development compared to tumorigenesis have similar components: invasion, reinitiation of cell proliferation, resistance to apoptosis, and angiogenesis.[21] These factors lead one to believe that the mammary gland is a good environment for cancer development. It is important to note; however, that obesity is generally considered an increased risk factor of breast cancer due to insulin resistance, especially in post-menopausal women. High circulating insulin levels promote DNA synthesis and cell proliferation, which can lead to cancer and metastasis.[22] These data elude to the idea that both sides of the spectrum leads to an increased risk for breast cancer; however, much more research will need to be conducted on this subject will be necessary to come to a sound conclusion on the relationship between Ube2k, adipogenesis and breast cancer.

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